

Indirect Reduction of Ear Molds and Associated Mycotoxins in *Bacillus thuringiensis* Corn Under Controlled and Open Field Conditions: Utility and Limitations

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ABSTRACT In 1995, ears of a experimental inbred (CG59-2) containing a synthetic *Bacillus thuringiensis* Cry IA(b) gene driven by PEPC, pith and pollen promoters and artificially infested with *Ostrinia nubilalis* (Hübner) larvae in small plot studies were free from insect damage, whereas 40–50% of the corresponding non-Bt inbred ears were damaged. Bt inbred ears that were inoculated with *Aspergillus flavus* Link and *Fusarium proliferatum* T. Matsushima (Nirenberg) or exposed to natural mold inoculum after infestation with *O. nubilalis* were free of visible signs of mold, as compared with ≈30–40% of the non-Bt ears similarly treated. Results in 1996 using the same inbred with a single allele dose of the Bt gene showed similar trends. Mean total fumonisin levels for non-Bt versus Bt inbred ears were not significantly different (2.8 versus 0.8 ppm, respectively) in 1996. In paired hybrid studies run in 0.4-ha (1-acre) fields, an event 176 Bt hybrid had significantly lower amounts of damage and signs of *Fusarium* spp. mold, but not fumonisin, compared with a corresponding non-Bt hybrid from 1996 to 1998. However, two hybrid pairs that contained either MON810 or Bt11 constructs examined in similar fields at the same site had lower levels of fumonisin in both 1997 (30- to 40-fold) and 1998. High intrafield variability in insect infestation and presence of *Helicoverpa zea* (Boddie) in Bt hybrids was apparently responsible for fewer significant differences in fumonisin levels in 1998. Similar trends for all three hybrid pairs were noted in small plot trials at another site. Incidence of other ear pests or insect predators varied as much among non-Bt hybrids as they did for Bt/non-Bt hybrid pairs.

KEY WORDS *Bacillus thuringiensis*, maize, *Zea*, *Ostrinia*, *Helicoverpa*, *Carpophilus*

MYCOTOXINS PRODUCED BY corn ear mold fungi cause hundreds of millions of dollar of direct and indirect costs per year in the United States (CAST 1989, USDA-ARS 1999). In corn destined for human consumption, acceptable levels for aflatoxins are from 0 to 20 parts per billion (ppb), whereas the proposed level for fumonisins in Europe is one part per million (ppm) (van Egmond 1998). It is well documented that ear feeding insects can significantly increase the incidence of these molds and associated toxins (for a review see Dowd 1998). Both insecticide treatments and use of tight-husked varieties of corn resistant to insects can significantly reduce the incidence of the molds and their toxins (Dowd 1998). However, current insecticide formulations usually cannot be applied economically to control insects well enough to reduce mycotoxin levels to acceptable levels as a result of the number of treatments required (for a review, Dowd 1998). Southern-adapted, tight husked germplasm does not yield as well as corn belt adapted germplasm (Jenkins 1947), and has increased susceptibility to

temperate ear molds caused by the extended drydown period in the field (Dowd 1998, Trenholm et al. 1989).

Recent genetic engineering technology has produced maize with near immunity to the European corn borer, *Ostrinia nubilalis* (Hübner), in tissue expressing 1,000+ ppm of Bt crystal protein in the soluble protein fraction (Kozziel et al. 1993). Expression of high levels of the *Bacillus thuringiensis* protein in the silks and ears of maize can greatly reduce damage by *O. nubilalis*. The reduced *O. nubilalis* damage should also reduce mold on the respective ears, as has been reported previously in small plot studies (Dowd et al. 1995, 1996; Munkvold et al. 1996, 1997, 1999; Munkvold and Hellmich 1998). Use of the Bt hybrids thus may not only help with insect management, but also with ear mold and mycotoxin levels. However, the number of other ear feeding pests that may be involved in mycotoxin production (Dowd 1998) and the potential concerns of Bt hybrid effects on nontarget and beneficial insects prompted comprehensive studies in Illinois. This report describes experiments in both small plot and in ≈0.4-ha (1-acre) fields that demonstrate reductions in the incidence and amount of ear damage caused by target caterpillars in Bt versus non-Bt inbreds or hybrids. These reductions in cater-

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pillar damage were often associated with both reductions in signs of mold and associated mycotoxins, such as fumonisins. This report also describes incidence and damage caused by nontarget insect pests of ears, and incidence of insect predators. Summaries of these studies have been reported previously (Dowd et al. 1995, 1996, 1997a, 1998a, 1999a).

Materials and Methods

Inbred Study. *Insects.* The *O. nubilalis* were obtained from either J. White, of Ciba Seeds (now Novartis), Bloomington, IL, or M. R. McGuire (USDA-ARS, National Center for Agricultural Utilization Research, Peoria IL). The colony obtained from M. R. McGuire was originally obtained from L. C. Lewis (USDA-ARS, Iowa State University). The Bloomington *O. nubilalis* were used for initial tests of seedlings in growth chambers in 1995, and the other *O. nubilalis* were used in all other studies.

Plants. Elite inbred seed (experimental inbred CG59-2 produced by Ciba Seeds [now Novartis]) segregating 1:1 for a Bt gene was used to obtain Bt corn in 1995. The construct in this inbred had a CryIA(b) synthetic Bt gene driven by PEPC, pith and pollen promoters, thus expression of the Bt protein was at high levels throughout most of the plant. A corresponding non-Bt inbred was used for comparative purposes. Because of unavailability of the same Bt seed in 1996, seed produced from the Bt \times non-Bt (pollen source) inbreds in 1995 was used. Seed was germinated in soil mix (Dowd 1994) and seedlings were grown in plant growth chambers (Dowd and Lagrimini 1997) at 27:21°C, 50% RH, and a photoperiod of 16:8 (L:D) h. Plants were transplanted into the field after three leaves were present. Initial fertilizer applications were the same as described previously (Dowd 1994), but were supplemented with Miracle Gro lawn food (Stern's Miracle-Gro Products, Port Washington, NY) (33% N, 6% P, 6% K) when pale leaves indicated signs of nitrogen stress. There were two supplemental treatments with fertilizer in 1995, and four in 1996. Flooding in 1996 killed most of the initial transplants, so new plants were started and transplanted.

Bacillus thuringiensis *Determination Assays for Experimental Inbreds.* In 1995, \approx 2 cm of the oldest leaf tip was removed from \approx 150 of the 1:1 segregating Bt seedlings and 75 of the non-Bt inbreds. Each leaf section was put in a Falcon 1006 petri dish (Becton Dickinson, Franklin Lakes, NJ) along with 10 neonate *O. nubilalis* larvae. The leaf sections were examined after 1 and 2 d for dead larvae and the degree of feeding damage. The source plants corresponding to leaf sections producing mostly dead larvae and almost no feeding after 2 d were considered Bt protein positive and were transplanted into the field. Of the seedlings tested, 50.3% were Bt protein positive.

Bacillus thuringiensis protein positive plants used in 1995 and 1996 were further checked for Bt protein expression just before field assays by collecting 2 by 2-cm sections of the terminal leaf tip of each plant in

the field. These leaves were evaluated Bt protein presence as just described.

Molds Used for Inbred Inoculation. The *Aspergillus flavus* Link strain used, NRRL 3357, was obtained from S. W. Peterson at the USDA-ARS National Center for Agricultural Utilization Research, Peoria, IL. This strain is a reliable aflatoxin producer often used in evaluating maize resistance to *A. flavus* and aflatoxin (Norton 1995). The *Fusarium proliferatum* (T. Matushima) Nirenberg strain used, M-5991, was obtained from G. A. Bennett (USDA-ARS National center for Agricultural Utilization Research, Peoria IL). This strain originally came from the Fusarium Research Center at The Pennsylvania State University. This strain is a reliable producer of fumonisins and moniliformin and was originally isolated from corn in Iowa (Javed et al. 1993). A separate spore suspension of each mold species was prepared at a concentration of 10^6 colony forming units per milliliter of 0.01% Triton \times 100. The spore suspensions were mixed 1:1 for application to ears.

Inbred Field Assays. Bt plants were detasseled during both 1995 and 1996 because of other corn being grown in the area, and pollen from the corresponding non-Bt inbred was used for all pollinations. Time of silking varied enough so that two series of ears were available each year, with pollination dates for each series varying by no more than 3 d. The most numerous plants (first series of pollinations in each case) were used in studies where both *O. nubilalis* and mold inoculum were added. Seven days after median silking of each series, 10 neonate *O. nubilalis* were individually transferred to the silks of each ear by using a probe. The caterpillars were confined to the ear tip by covering the ear tip with a 10 by 20-cm muslin bag, secured by a rubber band. One upper corner of the bag was cut off so an opening 1 cm wide was available. The open corner was folded over and held closed with a small binder clip.

Mold inoculum was added to the first series of ears 7 d after the caterpillars were added. The binder clip was removed and 0.5 ml of inoculum was added to the silks at the ear tip with an automatic pipet. The corner of the bag was folded back over and reclipped. Bags remained on those ears in the mold inoculation series until harvest. In the uninoculated series of experiments, the muslin bags were removed 1 wk after the *O. nubilalis* were added to permit naturally occurring molds to interact with the ears.

Evaluation of Inbred Ears. Ears were removed after black layering of kernels had occurred. Ears were examined for caterpillar damage and position of the damaged kernels on the ear. The number and type (filled or unfilled) of kernels damaged was also noted. Ears were also evaluated for signs of mold presence. The number and type of kernels, and the type of mold (if determinable) was recorded. Mold type was determined using guidelines provided in a corn disease manual (Shurtleff 1980) as described previously (Dowd et al. 1999). The association of mold with insect-damaged kernels was also recorded. Examinations for mold were done both by eye and under $40\times$

using a stereomicroscope. Ears were also examined under long wave (345 nm) UV light for the presence and numbers of kernels with bright greenish yellow fluorescence (BGYF) which is an indicator of *A. flavus* kernel invasion (e.g., Norton 1995).

Hybrid Studies. Hybrid Culture. Field scale experiments involved planting non-Bt/Bt pairs of Ciba 4494 and Max 454 (event 176) from 1996 to 1998, Pioneer P3394 and P33V08 (event MON810) and NK6800 and NK 6800 Bt (event Bt 11) from 1997 to 1998 at a research site near Kilbourne, IL (Dowd et al. 1998b). Each hybrid pair was planted in ≈ 0.4 -ha (1-acre) fields in four row alternating strips, with at least six strips of each hybrid type. Planting density and cultural conditions were the same as those described previously (Dowd et al. 1998b). Smaller paired experiments were also set up in Peoria, IL, in the same years, with at least 20 plants of each hybrid present and grown as described for inbreds.

Evaluation of Hybrid Ears. In fields near Kilbourne, IL, samples for first-generation *O. nubilalis* were taken once damage was noted in all 3 yr in the V9 to V12 stage. Sampling was performed as described previously (Dowd et al. 1998b) with about every fourth plant sampled in a center row of each strip, for a typical total of 25 plants. In 1996, an adjacent field of P3394 was also sampled in the same manner. In 1996 and 1997, the two axils below the ear axil of 25 plants in each row were sampled for insects (including predators), insect damage, and molds. Twenty-five milk stage ears per row were removed for evaluation in the laboratory for type and degree of insect damage as described previously, which included insect species present and numbers of kernels damaged by respective insects (Dowd et al. 1998). Harvest stage ears were sampled in the same manner as milk stage ears, and evaluated for amount and type of insect damage, numbers of kernels discolored, and numbers of kernels symptomatically molded.

During milk stage sampling in 1996, unexpectedly smaller *O. nubilalis* larvae were found on the Max 454 versus 4494 ears, so ear tissue from collected ears was tested for effects on *O. nubilalis*. For this experiment, 30 ear tips of each hybrid, ≈ 2 cm long, were removed from each ear and placed in 28-ml plastic cups. Five neonate *O. nubilalis* were added to each cup. Cups were held at $27 \pm 1^\circ\text{C}$, $40 \pm 10\%$ RH, and a photoperiod of 14:10 (L:D) h for 7 d, after which both mortality and weights of survivors were determined. The experiment was run in duplicate.

Mycotoxin Analyses. Insect-damaged kernels were separated from other kernels and both were analyzed separately for aflatoxins and fumonisins to increase the accuracy of the analysis. Data were recombined by weight for final mycotoxin level calculations. Prior work has indicated that insect-damaged kernels can comprise most of the source of fumonisin in an ear, in some cases having $>1,000$ ppm of fumonisins (Dowd et al. 1999). Samples were stored at -20°C until mycotoxin analysis for fumonisins and aflatoxins was performed by Romer Laboratories (Union, MO), using previously described procedures (Dowd et al. 1999).

Table 1. Effect of Bt gene in maize inbreds on *O. nubilalis* infestation, damage and mold in 1995

Parameter	Mold inoculum added		No mold inoculum added	
	Bt–	Bt+	Bt–	Bt+
<i>O. nubilalis</i>				
% infestation	53.2a	0.0b	41.5a	0.0b
% w/kernel damage	51.1a	0.0b	39.0a	0.0b
Mold				
% w/mold	38.3a	0.0b	29.3a	0.0b
% w/sporulation	0.0a	0.0b	0.0a	0.0a
% w/fluorescence	46.8a	10.0b	29.3a	3.2b

Mold inoculum was *A. flavus* and *F. proliferatum*. Respective number of ears are 47 and 30 for Bt– and Bt+ ears with inoculum added, and 41 and 31 for Bt– and Bt+ ears with no inoculum added. Values in rows of the same column grouping followed by different letters are statistically different at $P < 0.05$ by chi-square analysis.

High-performance liquid chromatography analysis detection limits were 1 ppb for aflatoxins B1, B2, G1, and G2; and 0.1 ppm for fumonisins B1, B2, and B3. Individual ears from inbred studies were tested for mycotoxins, whereas groups of three to five ears for small plot hybrid studies in Peoria were combined for analysis. All ears from each sampled row in hybrid studies at Kilbourne were pooled as an individual sample used for mycotoxin analysis.

Statistical Analyses. Significant differences in percent insect infestation and percent infection (molds) were determined by chi-square analysis due to non-normal distribution of some data, as has been described previously (Dowd et al. 1998b, 1999) using SAS PROC FREQ (SAS Institute 1987). Significant differences in numbers of kernels per ear that were insect-damaged or molded, and mycotoxin levels (after log transformation) were determined by analysis of variance (ANOVA) (PROC GLM, SAS Institute 1987). Correlation analysis was used to determine the degree of association between the insect damage, mold presence, and mycotoxins (PROC CORR, SAS Institute 1987).

Results

Inbred Studies. There was no indication of presence or ear damage by *O. nubilalis* larvae when they were added to the Bt inbred in 1995 (Table 1). Incidence of *O. nubilalis* on the non-Bt inbred ranged from ≈ 40 to 50%, with approximately the same percentage of ears sustaining *O. nubilalis* kernel damage whether they were inoculated with mold or not. None of the Bt ears had visible mold hyphae or sporulation regardless of whether they were inoculated with mold or not (Table 1). Although no visible mold was seen, 10% of the inoculated Bt ears had visible bright greenish yellow fluorescence (BGYF), typically on only one or two kernels per ear. In contrast, ≈ 30 –40% of the non-Bt ears had visible mold and up to 47% had BGYF, which was again limited to a few kernels per ear. Mold was associated with *O. nubilalis* damage 78% of the time when mold inoculum was added, and 94% of the time

Table 2. Effect of Bt gene (half level) in maize inbreds on *O. nubilalis* infestation, damage and mold in 1996

Parameter	Mold inoculum added		No mold inoculum added	
	Bt–	Bt+	Bt–	Bt+
<i>O. nubilalis</i>				
Tip/early damage				
% infestation	96.9a	33.3b	ND	ND
No. kernels/ear	9.4 ± 0.9a	9.3 ± 1.5a	ND	ND
Late damage				
% infestation	3.1a	19.0a	ND	ND
No. kernels/ear	6.0	8.8 ± 2.2	ND	ND
Side damage				
% infestation	31.2a	23.8a	ND	ND
No. kernels/ear	16.9 ± 4.8a	6.4 ± 3.1a	ND	ND
Overall				
% infestation	96.9a	52.4b	72.2a	0.0b
No. kernels/ear	9.3 ± 0.9a	9.1 ± 4.5a	19.0 ± 2.7	—
Mold				
Tip mold on filled kernels				
% incidence	46.8a	9.5b	ND	ND
No. kernels/ear	11.5 ± 2.5a	5.5 ± 1.5a	ND	ND
Tip mold on all kernels				
% incidence	70.6a	28.6b	ND	ND
No. kernels/ear	12.2 ± 1.5a	14.5 ± 2.9a	ND	ND
Mold on total ear on filled kernels				
% incidence	44.1a	19.0a	33.3a	0.0a
No. kernels/ear	11.3 ± 2.2a	6.8 ± 2.1a	11.2 ± 3.0	—
Mold on total ear on any kernels				
% incidence	88.2a	38.1b	60.0a	20.0a
No. kernels/ear	8.0 ± 0.6a	8.6 ± 1.2a	11.4 ± 2.5	4.0
Fumonisin, ppm	2.8 ± 1.3a	0.8 ± 0.3a	ND	ND

Mold inoculum was *A. flavus* and *F. proliferatum*. Respective number of ears are 32 and 21 for Bt– and Bt+ damage on inoculated ears, 5 and 18 for Bt– and Bt+ damage on noninoculated ears. Values reported for numbers of damaged kernels per ear are based only on ears where damage occurred. Values in rows for like column groupings followed by different letters are significantly different for incidence (chi-square analysis) and number of kernels/ear or fumonisin (ANOVA) at $P < 0.05$. ND, not determined due to low or no occurrence.

when no inoculum was added. Because ear fill was poor (apparently because of hot weather, which also adversely affected fill of other varieties being grown in the same location), no samples were submitted for mycotoxin analysis.

Heavy and repeated natural infestation by *O. nubilalis* in 1996 complicated interpretation of experi-

ments. Because of this natural infestation, location and type of infestation is distinguished in reported results. Early tip damage, as indicated by browning of the pericarp on damaged kernels, is attributed to those *O. nubilalis* specifically added to the ear tips and then covered by cloth bags. For those ears that had mold inoculum added, nearly 100% of the non-Bt ears had *O. nubilalis* damage at the tip, as compared with 33% of the Bt ears (Table 2). When caterpillar damage was present, the number of kernels damaged per ear at the tip was approximately the same for both inbreds. There was no significant difference in incidence of *O. nubilalis* that invaded through the ear side (which may result in higher rates of mold, Dowd et al. 1999) or invaded late (which typically results in little mold, Dowd et al. 1999) for the Bt versus non-Bt inbreds, and incidence was lower than for ear tips of the respective inbreds. Although when ear side damage occurred and the number of damaged kernels per ear was reduced by more than twofold for the Bt versus non-Bt corn, the differences were not significant. A more limited number of plants had natural mold invasion, but the amount of *O. nubilalis* infestation of all ear location types was still significantly less for the Bt compared with non-Bt corn (Table 2).

In 1996, the incidence of mold on the inbred ear tips where mold inoculum was purposely added was ≈ 4.5 –2.8-fold less for the Bt compared with non-Bt inbred, depending on whether only filled kernels or all kernels are considered (Table 2). When mold was present, the number of moldy filled kernels per ear was reduced by twofold for Bt versus non-Bt ears, but differences were not significant. At least a twofold reduction in the incidence of mold for Bt versus non-Bt ears was seen regardless of the location of the *O. nubilalis* damage on the ear (Table 2). Again, when mold was present, the number of filled moldy kernels per ear was reduced by twofold for the Bt compared to non-Bt corn over the entire ear. Similar trends were noted for the ears where mold inoculum was not added in 1996. Although mean total fumonisin levels were about threefold lower in Bt versus non-Bt ears, the differences were not significant. No ears had BGYF in 1996.

Hybrid Studies in 0.4-ha Fields. Control of first generation *O. nubilalis* by Bt hybrids was highly effective in all 3 yr at Kilbourne, IL (Table 3). When examined, a significantly higher percentage of non-Bt versus Bt plants had both mold and *O. nubilalis* larval

Table 3. Incidence of first-generation European corn borer-like damage at Kilbourne site on Bt and non-Bt hybrid pairs

Year	% infestation by <i>O. nubilalis</i>					
	Ciba		Pioneer		NK	
	4494 Bt–	Max 454 Bt+	3394 Bt–	33V08 Bt+	6800 Bt–	6800Bt Bt+
1996	16.0a	0.0b	36.5	NA	NA	NA
1997	86.0a	1.6b	82.4a	1.6b	80.0a	0.7b
1998	5.6a	0.0b	12.0a	0.0b	3.6a	0.0b

Number of ears for 1996 was 200. Number of ears for 1997 was 250 for Pioneer and Ciba series hybrids, 175 for NK6800 and 150 for NK6800Bt. Number of ears for 1998 was 150 and 160 for 4494, max454; 200 and 225 for 3394 and 33V08; 250 and 225 for NK6800 and NK6800Bt. Values in rows of like hybrid pairs followed by different letters are significantly different at $P < 0.05$ by chi-square analysis. NA, not available for that year.

Table 4. Distribution of insects and mold for axil samples (7-10 after pollination) at Kilbourne site in Bt and non-Bt hybrid pairs.

Organism	% incidence					
	Pioneer		Ciba		NK	
	3394 Bt-	33V08 Bt+	4494 Bt-	Max 454 Bt+	6800 Bt-	6800Bt Bt+
1996						
<i>Orius</i>	2.5	ND	26.0a	35.0b	ND	ND
<i>Fusarium</i>	88.0	ND	87.5a	86.0a	ND	ND
1997						
Sap beetles	8.8a	13.6a	1.6a	4.8a	3.4a	2.7a
<i>Orius</i>	6.8a	10.0a	3.2a	3.6a	3.4a	8.0a
Lady beetles	2.8a	1.6a	7.2a	9.6a	1.7a	2.0a
Aphids	NR	NR	20.4a	42.4b	8.0a	14.0a
<i>O. nubilalis</i>	36.8a	0.0b	42.8a	0.4b	29.1a	0.0b
<i>Fusarium</i>	75.2a	56.0b	74.0a	56.0b	70.9a	62.7a
<i>O. nubilalis</i> + <i>Fusarium</i>	33.2a	0.0b	35.2a	0.4b	27.4a	0.0b

ND, not determined; NR, less than 1% and not reported. Values in rows of like column groups followed by different letters are significantly different at $P < 0.05$ by chi-square analysis.

entry holes at the same axil in 1997 ($P = 0.000$, $\chi^2 = 99.520$). There were typically no differences in incidences of beneficial insects for Bt versus non Bt hybrids. However, there were several cases where the differences in incidence of predators were greater between different non-Bt hybrids than between Bt and non-Bt hybrid pairs (Table 4). The presence of lady beetles was relatively independent of aphid presence.

At milk stage, as expected, the Pioneer and NK Bt hybrid ears had no *O. nubilalis* damage, although damage by sap beetles (Coleoptera: Nitidulidae) and *Helicoverpa zea* (Boddie) did occur (Table 5). In 1998, it appeared infestation by second-generation *O. nubilalis* occurred later than normal. They were present fairly commonly in the Ciba series plants, but not the Pioneer or NK series plants. The environmental conditions apparently delayed development of the Ciba

Table 5. Distribution of insects for milk stage samples at Kilbourne site on Bt and non-Bt hybrid pairs

Organism	Pioneer		Ciba		NK	
	3394 Bt-	33V08 Bt+	4494 Bt-	Max 454 Bt+	6800 Bt-	6800Bt Bt+
1996						
% ECB	13.2b	ND	28.9a	21.8a	ND	ND
ECB kd/ear	6.2 ± 1.5	ND	8.9 ± 0.8a	1.3 ± 0.2b	ND	ND
% CEW	4.9	ND	8.2a	7.4a	ND	ND
CEW kd/ear	43.5 ± 6.1	ND	40.6 ± 4.6a	33.7 ± 4.9b	ND	ND
% Orius	14.2	ND	20.6a	18.8a	ND	ND
% Aphids	0.0	ND	15.5a	10.4a	ND	ND
1997						
% ECB	76.8a	0.0b	79.3a	76.8a	80.6a	0.0b
ECB kd/ear	7.0 ± 0.4	—	see below		10.5 ± 0.8	—
% CEW	1.4a	3.5a	1.5a	0.5a	0.6a	0.0a
CEW kd/ear	15.3 ± 5.9a	3.0 ± 0.4b	17.3 ± 7.9	—	23.0	—
% SB	5.3a	2.5a	8.4a	4.4a	6.9a	8.0a
SB kd/ear	3.7 ± 1.6a	3.0 ± 0.5a	3.8 ± 1.2a	2.7 ± 0.9a	—	7.0 ± 2.3
% Orius	37.2a	53.2b	44.8a	50.7a	40.6a	50.0a
% lady beetles	2.4a	3.0a	5.4a	2.5a	7.4a	1.3b
% carabid larva	7.7a	8.0a	4.9a	9.9a	1.7a	0.7a
% spiders	14.0a	12.4a	12.3a	14.3a	12.0a	8.0a
Ciba specific						
% recovery ECB from ears			73.9a	53.2b		
% ears with 2 or more ECB			30.4a	14.5b		
Mean ECB size, mm			9.5 ± 0.4a	5.5 ± 0.3b		
No. kernels damage by ECB/ear			10.1 ± 0.8a	1.3 ± 0.2b		
1998						
% ECB	0.0a	0.0a	38.8a	24.8b	0.0a	0.0b
ECB kd/ear	—	—	10.9 ± 0.9a	7.0 ± 0.9b	—	—
% CEW	2.0a	0.0a	0.0a	0.0a	7.4a	12.4a
CEW kd/ear	12.5 ± 2.5	—	—	—	29.3 ± 2.2a	10.7 ± 1.7b
% SB	10.0a	8.0a	30.8a	23.2b	3.4a	2.5a
SB kd/ear	4.2 ± 1.0a	3.8 ± 1.1a	3.4 ± 0.5a	3.5 ± 0.5b	2.0	4.0 ± 2.5

ECB, *O. nubilalis*; CEW, *H. zea*; SB, sap beetle; kd, kernels damaged. Values in rows of like column pairs followed by different letters are significantly different at $P < 0.05$ by analysis of variance (number of kernels damaged) or chi-square analysis (incidence).

series plants by about a week, despite being planted on the same day. This difference in developmental rate contrasted with what was seen in 1997 for this same group of hybrids, because silking occurred on approximately the same days. When corresponding values were present (one instance each in 1996, 1997, and 1998), the numbers of kernels damaged by *H. zea* larvae were significantly less for Bt versus non-Bt hybrids. Again, when beneficial insects were monitored, their incidence was typically very similar for Bt and non-Bt pairs, and varied more between different hybrids of the same Bt type than for Bt and non-Bt pairs. At milk stage, the numbers of Max 454 kernels damaged per ear by *O. nubilalis* was 6.5 (1996) to eightfold (1997) less than the 4494 non-Bt hybrid. The dramatic reduction in *O. nubilalis* damage on Max 454 compared with 4494 ears at milk stage was unexpected, based on the low level of Bt protein previously reported for the silk and kernels. Laboratory studies of ear tips from ears sampled in 1996 without filled kernels (the state they were in when ears were sampled at milk stage), indicated higher mortality of *O. nubilalis* larvae after 1 wk for those fed on the Bt ear tips compared with the non-Bt ear tips (34.4 and 1.5% for set 1, and 25.7 and 0.78% for set 2, respectively). The Bt ear tips also caused significant reductions in growth rates of *O. nubilalis* larvae compared with non-Bt ear tips as reflected by weights of survivors (0.53 ± 0.06 versus 5.89 ± 0.24 mg, $P = 0.000$, $F = 221.246$, $df = 1$, 192 for set 1; and 0.78 ± 0.08 versus 4.52 ± 0.26 mg, $P = 0.000$, $F = 134.423$, $df = 1$, 183 for set 2, respectively).

At harvest, kernels damaged by *O. nubilalis*, whether early (discolored pericarp) or late in occurrence, were typically lower in number for Bt versus non-Bt hybrids (Table 6). When primarily *H. zea* damage was present, there was no significant difference in numbers of kernels damaged by *H. zea* in Bt versus non-Bt ears, even in the Bt hybrids that expressed the protein throughout the plants. Incidence of ears with sap beetle damage was significantly higher on the Pioneer ($P = 0.001$, $\chi^2 = 11.083$) and Ciba ($P = 0.004$, $\chi^2 = 8.189$) Bt compared to non-Bt hybrids in 1997. In 1997, at milk stage, sap beetles were frequently found feeding on caterpillar damaged kernels (which occurred at a high rate) in the non-Bt ears. In the milk stage Bt ears, which had much lower caterpillar damage, it was more common to see sap beetle adults and larvae causing the initial damage of the kernels. This trend was apparently reflected at harvest, as well. There was a higher incidence of stink bug (Hemiptera: Pentatomidae) damage for some Bt hybrids compared with non-Bt hybrids in 1997 but not 1998. However, stink bug incidence of damage varied as much between different non-Bt hybrids as for Bt/non-Bt hybrid pairs. Ears with "popped" kernels (kernels with split pericarps and visible expanded endosperms) were more common for all hybrids in 1997 than 1998, but incidence and degree of damage between different Bt/non-Bt hybrid pairs was not significantly different for any year/hybrid pair combination.

At harvest, significant reductions in incidence of visibly molded kernels damaged by caterpillars was

noted for Ciba Max 454 versus 4494 ears in all 3 yr at Kilbourne, but fumonisin levels were only reduced significantly for Bt versus non-Bt hybrids in 1998 ($P = 0.028$; $df = 1$, 11; $F = 6.228$) (Table 6). Significant reductions in mold incidence and fumonisin levels were noted for Bt versus non-Bt P3394 ($P = 0.001$; $df = 1$, 14; $F = 20.458$) and NK6800 ($P = 0.000$; $df = 1$, 11; $F = 40.090$) pairs in 1997, but differences were less dramatic in 1998, and fumonisin levels were not significantly different between Bt and non-Bt hybrid pairs. When all three fumonisins were detected, total fumonisins were generally comprised of $\approx 75\%$ fumonisin B1, 20% fumonisin B2, and 5% fumonisin B3. When present, caterpillars were the major contributors of visibly molded kernels, but when *H. zea* were absent in 1997, sap beetle-damaged kernels and popped kernels could contribute the major amount of visibly molded kernels for the Bt hybrids. No aflatoxins were detected from the Kilbourne samples in any years.

Both incidence of visibly molded kernels and fumonisin were typically significantly correlated with numbers of insect-damaged kernels in all years, although higher correlations were typically noted between numbers of visibly molded and insect-damaged kernels. (Table 7). Correlations between insect damage and fumonisins were typically not significant for small plot studies (data not shown).

Hybrid Studies in Small Plots. The same trend in fumonisin levels noted at Kilbourne for the Ciba hybrid pairs was seen in small plot studies in Peoria. In 1996, total fumonisin levels for non-Bt versus Bt hybrid ears were 8.8 ± 6.0 ppm (two pooled samples) versus 2.0 ppm (single pooled sample) for ears inoculated with caterpillars and spore suspensions (as for inbreds), and <0.35 ppm fumonisin for either type when uninoculated. In 1997, total fumonisin levels for non-Bt versus Bt ears were 4.8 ± 1.9 versus 2.8 ± 1.5 ppm, respectively ($P = 0.555$). Fumonisin levels were not determined in 1998 because of low incidence of insect damage for both hybrids. Results for small plot trials in Peoria with the NK and Pioneer hybrid pairs generally reflected the trends noted for the larger area trials in Kilbourne. Total fumonisins were eightfold higher in the non-Bt versus Bt NK6800 hybrid pairs in 1997 (1.3 ± 0.3 and 0.16 ± 0.13 ppm, respectively, $P = 0.012$; $df = 1$, 6; $F = 12.699$), and a similar effect was noted in 1998 (0.22 ± 0.08 and 0.03 ± 0.02 ppm, respectively, $P = 0.001$; $df = 1$, 14; $F = 17.024$). The Pioneer pair was not planted at Peoria in 1997, and for 1998 fumonisin levels were 0.055 ± 0.025 and 0.256 ± 0.147 ($P = 0.503$) for the non-Bt/Bt hybrid pairs, respectively. For the 1998 Pioneer samples, *H. zea* was more common on the 33V08 than 3394, and a few ears of the 33V08 additionally had symptomatic infected kernels not associated with insect damage. Total fumonisins were typically composed of the same percentage of the three fumonisins as found for samples from the Kilbourne site. Aflatoxin was rarely encountered, and only occurred for a few ear groups at Peoria in 1998. There were no significant differences in af-

Table 6. Distribution of insect damage, mold and mycotoxins for harvest samples at Kilbourne on Bt and non-Bt hybrid pairs

Organism	Pioneer		Ciba		NK	
	3394 Bt–	33V08 Bt+	4494 Bt–	Max 454 Bt+	6800 Bt–	6800Bt Bt+
1996						
% early CAT kd	4.1	ND	50.5a	26.4b	ND	ND
% >10 kd/ear	1.7	ND	31.8a	9.3b	ND	ND
No. kd/ear	10.6 ± 4.3	ND	15.3 ± 1.1a	9.5 ± 1.0b	ND	ND
% late CAT kd	40.7	ND	63.5a	61.7a	ND	ND
% > 10 kd/ear	29.1	ND	41.2a	19.7b	ND	ND
No. kd/ear	19.6 ± 1.6	ND	17.4 ± 1.2a	9.5 ± 0.7b	ND	ND
% cat + mold	0.6	ND	37.0a	15.0b	ND	ND
No. kd/ear	3.0	ND	8.4 ± 1.0a	3.2 ± 0.4b	ND	ND
% SB kd	0.6	ND	7.2a	6.2a	ND	ND
No. kd/ear	1.0	ND	2.1 ± 0.5a	1.8 ± 0.4a	ND	ND
% SB + mold	0.0	ND	5.2a	2.6a	ND	ND
No. kd/ear	—	ND	2.5 ± 0.5a	2.4 ± 0.9a	ND	ND
% STB kd	1.2	ND	1.6a	4.2a	ND	ND
No. kd/ear	19.5 ± 8.5	ND	5.3 ± 3.4a	4.5 ± 1.6a	ND	ND
% pop kd	3.5	ND	0.0a	0.0a	ND	ND
No. pop kd/ear	3.7 ± 1.2	ND	—	—	ND	ND
% pop + mold	0.6	ND	—	—	ND	ND
No. kd/ear	1.0	ND	—	—	ND	ND
% mold kd	1.2	ND	37.0a	20.2b	ND	ND
No. kd/ear	2.0 ± 1.0	ND	8.4 ± 1.0a	3.2 ± 0.4b	ND	ND
% > 10 kd/ear	0.0	ND	8.9a	0.5b	ND	ND
Fumonisin, ppm	ND	ND	0.89 ± 0.28a	0.52 ± 0.20a	ND	ND
1997						
% early CAT kd	80.0a	0.0b	90.5a	58.5b	64.0a	0.0b
No. kd/ear	13.7 ± 0.6	—	15.0 ± 0.7a	7.9 ± 0.5b	7.1 ± 0.4	—
% late CAT kd	73.5a	7.5b	92.5a	78.5b	66.9a	2.0b
No. kd/ear	18.5 ± 1.0a	10.6 ± 2.4b	25.8 ± 1.2a	13.2 ± 0.8b	9.5 ± 0.7	—
% total CAT kd	91.0a	7.5b	96.5a	84.0b	84.0a	2.7b
% CAT + mold	1.8a	0.01a	3.7a	0.8a	1.7a	0.0a
No. kd/ear	3.4 ± 0.2	2.0	5.4 ± 0.4a	2.6 ± 0.3b	4.3 ± 0.3	—
% SB kd	14.0a	27.5b	14.5a	26.0b	10.9a	18.0a
No. kd/ear	1.6 ± 0.2a	2.5 ± 0.2a	2.4 ± 1.0a	1.7 ± 0.2a	1.7 ± 0.3a	2.2 ± 0.3a
% SB + mold	3.0a	8.5a	5.0a	3.5a	4.0a	6.0a
No. kd/ear	1.2 ± 0.2a	1.8 ± 0.2a	3.0 ± 1.7a	1.7 ± 0.2a	1.7 ± 0.3a	1.4 ± 0.3a
% STB kd	24.0a	46.0b	14.0a	25.5b	46.9a	40.7a
No. kd/ear	8.1 ± 0.9a	6.5 ± 0.6a	6.1 ± 1.1a	5.8 ± 0.7a	7.2 ± 0.6a	5.8 ± 0.7a
% pop kd	22.0a	27.0a	12.5a	13.0a	33.1a	33.1a
No. kd/ear	3.5 ± 0.5a	2.7 ± 0.3a	2.0 ± 0.3a	2.1 ± 0.3a	3.5 ± 0.4a	3.0 ± 0.3a
% pop + mold	2.5a	2.5a	1.5a	1.5a	3.0a	3.5a
No. kd/ear	1.2 ± 0.2a	1.0 ± 0.0a	1.3 ± 0.3a	2.0 ± 0.0a	1.5 ± 0.3a	2.4 ± 1.0a
% insect kd	97.5a	74.0b	98.5a	92.5a	95.4a	68.0b
% mold kd	58.0a	8.5b	72.0a	38.5b	42.3a	11.3b
Fumonisin, ppm	1.1 ± 0.3a	0.028 ± 0.034b	2.6 ± 0.5a	3.5 ± 0.7a	1.4 ± 0.4a	0.049 ± 0.025b
1998						
% early CAT kd	—	—	43.7a	31.8b	—	—
% ECB kd	17.0a	0.0b	74.7a	56.3b	20.5a	0.0b
% CAT kd	31.5a	12.5b	74.8a	56.3b	45.0a	6.0b
No. kd/ear	8.0 ± 1.3a	8.9 ± 2.4a	12.4 ± 1.1a	9.9 ± 2.1a	6.7 ± 0.6a	6.8 ± 1.6a
Type kd	early and late		early only		early and late	
% CAT + mold	2.5a	0.0b	18.5a	7.3b	10.5a	2.0b
No. kd/ear	2.4 ± 0.8	—	4.0 ± 0.6a	3.2 ± 1.0a	4.0 ± 0.6a	3.2 ± 1.0a
% SB kd	8.5a	6.5a	13.9a	7.9a	11.0a	4.5b
No. kd/ear	1.4 ± 0.1a	1.3 ± 0.1a	1.6 ± 0.4a	2.4 ± 0.7a	1.8 ± 0.5a	2.2 ± 0.4a
% SB + mold	1.0a	0.5a	6.0a	2.0a	3.0a	2.0a
No. kd/ear	1.5 ± 0.5a	1.5 ± 0.5a	1.0 ± 0.0a	1.1 ± 1.1a	1.0	2.2 ± 0.6
% STB kd	18.5a	14.0a	4.5a	1.3a	7.0a	4.0a
No. kd/ear	4.4 ± 0.5a	4.7 ± 0.6a	9.0 ± 1.0a	4.8 ± 1.5a	4.2 ± 1.0a	4.7 ± 1.0a
% pop kd	12.0a	11.5a	4.0a	1.3a	1.5a	4.0a
No. kd/ear	2.8 ± 0.4a	2.9 ± 0.5a	1.5 ± 0.5a	2.0 ± 0.8a	1.1 ± 0.1a	1.3 ± 0.3a
% insect kd	65.5a	38.5b	82.8a	62.2b	54.5a	15.0b
% mold kd	3.5a	1.0a	18.5a	7.3b	12.0a	3.0b
Fumonisin, ppm	0.02 ± 0.03a	0.01 ± 0.03a	1.4 ± 0.4b	0.4 ± 0.1a	0.2 ± 0.1a	0.05 ± 0.1a

In 1998, essentially all damage was late for NK6800 series and P3394 series, but values for 4494/max 454 early and damage are 43.7 versus 31.8, and 44.4 versus 35.1, respectively, with only early damage significantly different at $P < 0.05$. kd, kernel damage; CAT, caterpillar; ECT = *O. nubilalis*; SB, sap beetle; STB, stink bug (Pentatomidae); pop, popped kernels; mold, *Fusarium*. ND, not determined. Values in rows of like column pairs followed by different letters are statistically different at $P < 0.05$ by ANOVA (kernel numbers) or chi-square analysis (percent incidence).

Table 7. Correlations of numbers of insect damaged kernels in samples, visibly molded kernels, and fumonisin levels at Kilbourn on Bt and non-Bt hybrid pairs

Parameters	Insect damaged kernels	Visibly molded kernels	Total fumonisins levels, ppm
	1996—4494/Max454		
Insect damaged kernels	—	0.85*	0.50
Visibly molded kernels	—	—	0.44
	1996—P3394		
Insect damaged kernels	—	ND	ND
Visibly molded kernels	—	—	ND
	1997—4494/Max454		
Insect damaged kernels	—		Both 4494 Max 454
Visibly molded kernels	—	0.82*	0.10 0.87* 0.78*
	1997—P3394/33VO8		
Insect damaged kernels	—	0.96*	0.81*
Visibly molded kernels	—	—	0.75*
	1997—NK6800/6800Bt		
Insect damaged kernels	—	0.93*	0.71*
Visibly molded kernels	—	—	0.69*
	1998—4494/Max454		
Insect damaged kernels	—	0.61*	0.60*
Visibly molded kernels	—	—	0.61*
	1998—P3394/33VO8		
Insect damaged kernels	—	0.86*	0.54*
Visibly molded kernels	—	—	0.50*
	1998—NK6800/6800Bt		
Insect damaged kernels	—	0.73*	0.66*
Visibly molded kernels	—	—	0.89*

ND, not determined. Correlation values followed by an * are significant at $P < 0.05$.

latoxin levels between Bt and non-Bt ears for these hybrids.

Discussion

Associations Between Reduction of Insect Damage and Mycotoxigenic Molds. There was a consistent reduction in both *O. nubilalis* incidence and damage, and associated ear mold incidence and kernel infestation rates for the Bt inbred compared with the non-Bt inbred in both years. Because the seed used in 1996 had only one Bt allele in maternal tissue, pollination with pollen from the non-Bt line would theoretically result in several different tissues that would contain lower amounts of Bt crystal protein than in 1995. This may help explain the more limited efficacy of the Bt inbred for *O. nubilalis* in 1996 compared with the 1995 study.

The commercially available Bt hybrid that expressed only limited amounts of Bt crystal protein in the kernel (Max 454) was not very effective in preventing *O. nubilalis* infestation of ears compared with the Bt hybrids that expressed the protein at high levels in ears and silks, although numbers of kernels damaged per ear by *O. nubilalis* and the incidence of symptomatic ear mold was still significantly less than for the corresponding non-Bt hybrid (Dowd et al. 1996, 1997a, and current data). Corn hybrids with other Bt genes grown in Puerto Rico and Hawaii had lower rates of insect damage (predominately *H. zea*) and ear

mold compared with corresponding non-Bt hybrids (Dowd et al. 1997a). Thus, significant reductions of insect damage and associated ear molds appears consistent regardless of the source gene or location (although the degree varies), but relative reductions in fumonisins were variable and depended on the degree of insect infestation (and associated mold) and expression pattern of the gene. Similar trends for plants expressing the Bt protein throughout the plant versus mainly in green tissue have been noted for fumonisin in small plot studies in Iowa, although in several cases hand inoculation with *F. moniliforme* and *O. nubilalis* either produced or accentuated differences in fumonisin levels for Bt versus non-Bt hybrids compared with natural conditions (Munkvold et al. 1999). Fumonisin reductions in Bt versus non-Bt corn have also been mentioned in North Carolina (van Duyn 1998). However, aflatoxin in a Bt hybrid was not greatly reduced compared with the non-Bt hybrid in Mississippi for all treatments, although significant insect damage of Bt ears did occur (Windham et al. 1999) (perhaps because of *H. zea* presence). Although of limited occurrence in the current study, signs of *A. flavus* and aflatoxin also were more similar between Bt and non-Bt plants compared with fumonisin. Overall, it appears that for effective reduction in mycotoxins in corn ears, the ears need to be essentially free of insect damage. A single damaged kernel, if molded, can potentially serve as a continuing source of mold to the rest of the undamaged kernels.

Environmental Influences on Efficacy of Insect Control in Indirectly Reducing Mycotoxins. Environmental conditions, interacting with hybrid type, can also apparently mitigate the efficacy of the Bt resistance in indirectly reducing mycotoxins. In studies using insecticides, insect damage was significantly correlated with fumonisins for the same hybrid in some years but not others, and two different hybrids varied in the strength of the correlation in the same year (Dowd et al. 1999). Efficacy of Bt hybrids in indirectly reducing fumonisins was also severely affected by large numbers of *H. zea* present in commercial fields evaluated in 1998 and 1999, with little effect on incidence and numbers of damaged kernels and fumonisin levels only reduced by approximately twofold (Dowd et al. 1998a, 1999; P.F.D., unpublished data). However, high and significant correlations between numbers of insect-damaged kernels and fumonisin levels still occurred (Dowd et al. 1998a, 1999; P.F.D., unpublished data). Damage by *H. zea* and *Carpophilus* sap beetles also appeared to contribute some kernels high in fumonisin in the current study. Event 176 hybrids tested against *H. zea* were not effective in significantly reducing ear damage (Pilcher et al. 1997b). Those hybrids expressing the gene throughout the plant, such as MON810 or Bt 11 events, have provided nearly complete control of *H. zea* in ears in some cases (Rice and Pilcher 1998), but <90% control has been reported in other cases (Gould 1998). More than 10-fold variations in susceptibility of *H. zea* to *B. thuringiensis* has been reported for different geographic populations (Stone and Sims 1993), which may explain the differences in reported efficacy of Bt hybrids against *H. zea*.

Comparison of Methods of Insect Control and Associated Reductions of Mycotoxins. Insect facilitation of mycotoxigenic fungal invasion is well established for several different crops, including corn, and can involve several different insect species (Dowd 1998). All insects associated with ear mold in the current study have previously been reported in the same role (Dowd 1998). There have been reports of stink bug (Pentatomidae) associations with mycotoxigenic fungi in some cases in the past (Dowd 1998), but there was no obvious association in the current study.

Although several applications may be needed, insecticide treatments have generally reduced both insect damage and associations of mycotoxigenic fungi (Smeltzer 1959, Anderson et al. 1975, Lillehoj et al. 1976, Smith and Riley 1992, Dowd et al. 1995). An exception to this trend occurred when very low levels of mycotoxins were present (Widstrom et al. 1976). Tight husks with long silk channels can also significantly reduce insect damage, *Aspergillus flavus*, and aflatoxin in corn (McMillian et al. 1985, 1987, Barry et al. 1986). However, germplasm from which these lines were derived is typically lower yielding (Jenkins 1947). Slower drydown rates can promote incidence of *Fusarium* molds in more northerly corn growing areas (Trenholm et al. 1989), and presumably fumonisin as well.

Bacillus thuringiensis corn is reportedly more effective in controlling *O. nubilalis* than are conventional insect sprays (Mycogen Plant Sciences 1996), and also has higher yields (because of yield protection) than corresponding non-Bt lines (Ciba 1995). Yields ("yield protection") can surpass the highest yielding unrelated non-Bt lines (Mycogen Plant Sciences 1996). Bt corn seed costs from \$5 to \$12 more per acre, but economic benefits can be seen if there are one or more corn borers per plant (Rice and Pilcher 1998). Although there are concerns for insect resistance development (Roush 1997, Gould 1998) adoption of Bt corn, while not a cure-all for mycotoxigenic ear molds, should at least not increase, and is likely to significantly decrease, the incidence of mycotoxins in corn. Although nontarget effects on other insects in laboratory studies have generated some concern (Losey et al. 1999), results of past (Pilcher et al. 1997a) and the current study indicate limited effects of Bt corn on beneficial insects. Relative levels of efficacy of Bt corn in indirectly reducing mycotoxins will, however, depend on the composition of ear damaging insect species present, and other hybrid-environment interactions that may undermine potential advantages achieved by reduction of insect damage in Bt hybrids. Although some hybrids that express the Bt crystal protein throughout the plant have thus far had significant correlations between numbers of insect-damaged kernels and fumonisin levels, others have not (P.F.D., unpublished data). Where colonization by mycotoxigenic fungi is primarily insect dependent, similar decreases in mycotoxin levels would be expected for other crops, such as cotton seed, peanuts, and tree nuts (Dowd 1998). Reductions of aflatoxin levels in cotton seed from Bt versus non-Bt cotton have already been demonstrated (Kishore 1995, Cotty et al. 1997). Further development of crops that are highly insect resistant because of other mechanisms would also be expected to significantly reduce associated mycotoxin problems when fungal invasion is primarily dependent on insect damage, and the environment favors plant health over fungal inoculum.

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